09/355793

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address ASSISTANT COMMISSIONER FOR PATENTS
Box PCT
Washington, D C 20231

US APPLICATION NO		FIRST NAMED APPLICANT	ATTY DOCKET NO
09/355,793		BLASER M	15979

INTERNATIONAL APPLICATION NO
PCT/US98/01730

I A FILING DATE	PRIORITY DATE
01/30/98	00/00/00

DATE MAILED 09/03/99

5611

MCGREGOR & ADLER
8011 CANDLE LANE
HOUSTON TX 77071

NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371 IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

1. The following items have been submitted by the applicant or the IB to the United States Patent and Trademark Office as ☐ a Designated Office (37 CFR 1.494), ☒ an Elected Office (37 CFR 1.495):

- ☒ U.S. Basic National Fee.
- ☒ Copy of the international application in:
  - ☐ a non-English language.
  - ☒ English.
- ☐ Translation of the international application into English.
- ☐ Oath or Declaration of inventors(s) for DO/EO/US.
- ☐ Copy of Article 19 amendments.
- ☐ Translation of Article 19 amendments into English.
- ☒ The International Preliminary Examination Report in English and its Annexes, if any.
- ☐ Translation of Annexes to the International Preliminary Examination Report into English.
- ☐ Preliminary amendment(s) filed _____ and _____.
- ☐ Information Disclosure Statement(s) filed_____ and _____.
- ☒ Assignment document.
- ☐ Power of Attorney and/or Change of Address.
- ☐ Substitute specification filed _____.
- ☒ Statement Claiming Small Entity Status.
- ☐ Priority Document.
- ☒ Copy of the International Search Report ☐ and copies of the references cited therein.
- ☐ Other:

2. The following items **MUST** be furnished within the period set forth below in order to complete the requirements for acceptance under 35 U.S.C. 371:

- ☐ a. Translation of the application into English. Note a processing fee will be required if submitted later than the appropriate 20 or 30 months from the priority date.
  - ☐ The current translation is defective for the reasons indicated on the attached Notice of Defective Translation.
- ☐ b. Processing fee for providing the translation of the application and/or the Annexes later that the appropriate 20 or 30 months from the priority date (37 CFR 1.492(f)).
- ☐ c. Oath or declaration of the inventors, in compliance with 37 CFR 1.497(a) and (b), identifying the application by the International application number and international filing date.
  - ☒ The current oath or declaration does not comply with 37 CFR 1.497(a) and (b) for the reasons indicated on the attached PCT/DO/EO/917.
- ☒ d. Surcharge for providing the oath or declaration later that the appropriate 20 or 30 months from the priority date (37 CFR 1.492(e)).

3. Additional claim fees of $_____ as a ☐ large entity ☐ small entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due (37 CFR 1.492(g)). See attached PTO-875.

ALL OF THE ITEMS SET FORTH IN 2(a)-2(d) AND 3 ABOVE MUST BE SUBMITTED WITHIN ONE MONTH FROM THE DATE OF THIS NOTICE OR BY ☐ 21 OR ☒ 31 MONTHS FROM THE PRIORITY DATE FOR THE APPLICATION, WHICHEVER IS LATER. FAILURE TO PROPERLY RESPOND WILL RESULT IN ABANDONMENT.

The time period set above may be extended by filing a petition and fee for extension of time under the provisions of 37 CFR 1.136(a).

4. Translation of the Annexes MUST be submitted no later that the time period set above or the annexes will be cancelled. Note processing fee will be required if submitted later than 30 months from the priority date.

5. ☐ The Article 19 amendments are cancelled since a translation was not provided by the appropriate 20 (37 CFR 1.494(d)) or 30 (37 CFR 1.495(d)) months from the priority date.

Applicant is reminded that any communication to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above. (37 CFR 1.5)

A copy of this notice MUST be returned with this response.

Enclosed: ☐ PCT/DO/EO/917 ☐ Notice of Defective Translation ☐ PTO-875

Deborah Williams
National Stage Processing
Telephone (703) 305-3744

FORM PCT/DO/EO/905 (December 1997)

09/355793

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: ASSISTANT COMMISSIONER FOR PATENTS
Washington, D.C. 20231

U.S. APPLICATION NO		FIRST NAMED APPLICANT	ATTY. DOCKET NO
09/355,793		BLASER	M DS979

INTERNATIONAL APPLICATION NO
PCT/US98/01780

I A FILING DATE	PRIORITY DATE
01/30/98	00/00/00 09/03/99

DATE MAILED

MCGREGOR & ADLER
8011 CANDLE LANE
HOUSTON TX 77071

5611

NOTIFICATION OF A DEFECTIVE OATH OR DECLARATION

This application fails to contain an oath or declaration acceptable under 35 U.S.C. 371 (c)(4) for entry into the national stage in the United States of America. The period within which to correct these requirements and avoid abandonment is set in the accompanying Office action.

A new oath or declaration, identifying this application by the international application number and international filing date is required. The oath or declaration does not comply with 37 CFR 1.497(a) and (b) in that it:

1. ☒ is not executed in accordance with either 37 CFR 1.66 or 37 CFR 1.68.
2. ☐ does not identify the specification to which it is directed.
3. ☐ does not identify the inventor(s).
4. ☐ does not identify the citizenship of each inventor.
5. ☐ does not state the person making the oath or declaration believes the named inventor or inventors to be the original and first inventor or inventors of the subject matter which is claimed and for which a patent is sought.

FAILURE TO SUBMIT AN OATH OR DECLARATION IN COMPLIANCE WITH 37 CFR 1.497(a) AND (b) WITHIN THE TIME PERIOD SET WILL RESULT IN FAILURE TO ENTER THE NATIONAL STAGE AND THE ABANDONMENT OF THE APPLICATION.

Additionally, the oath or declaration does not comply with 37 CFR 1.63 in that it:

1. ☐ does not identify the city and state or city and foreign country of residence or each inventor.

2. ☐ does not state that the person making the oath or declaration:

   a. ☐ has reviewed and understands the contents of the specification, including the claims, as amended by any amendment specifically referred to in the oath or declaration.

   b. ☐ acknowledges the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

3. ☐ does not identify the foreign application for patent or inventor's certificate on which priority is claimed pursuant to 37 CFR 1.55, and any foreign application having a filing date before that of the application on which priority is claimed, by specifying the application serial number, country, day, month, and year of its filing.

4. ☐ does not state that the person making the oath or declaration acknowledges the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and filing date of the continuation in part application which discloses and claims subject matter in addition to that disclosed in the prior application (37 CFR 1.63(d)).

Deborah Williams
National Stage Processing
(703) 305-3744

Telephone: (703)

Applicant or Patentee: Blaser, M. Docket No.: D5979
Attorney's Serial or Patent No.:
Filed or Issued: January 30, 1997
For: ~~Means For~~ Method of *Delivering Antigens For Vaccination with a Live Vector*

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(c)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official of the nonprofit organization empowered to act on behalf of the concern identified below:

NAME OF ORGANIZATION: **Vanderbilt University**
ADDRESS OF CONCERN: **405 Kirkland Hall**
Nashville, Tennessee 37240

TYPE OF ORGANIZATION:

X University of other institution of higher learning.

I hereby declare that the above identified nonprofit organization qualifies as a nonprofit organization as defined in 37 CFR 1.9(e), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization identified above with regard to the invention, entitled **as named above** by inventor(s) **as named above** described in the specification filed herewith.

If the rights held by the above identified nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Name
Address

[ ]Individual [ ] Small Business Concern [X]Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name Of Person Signing: LARRY R. STERANKA
Title Of Person Other Than Owner: _____
Address Of Person Signing: 1110 21st Ave. South, Suite 850, Nashville, Tennessee 37203
Signature: Larry R. Steranka
Date: 1/30/97

METHOD OF DELIVERING ANTIGENS FOR VACCINATION WITH A LIVE VECTOR

BACKGROUND OF THE INVENTION

Federal Funding Legend

This invention was supported in part under grant RO1-AI24145 from the National Institutes of Health. Accordingly, the United States government has certain rights in this invention.

Field of the Invention

The present invention relates generally to the fields of immunology and vaccine technology. More specifically, the present invention relates to methods of delivering antigens for vaccination with a live vector.

Description of the Related Art

One way in which microorganisms can alter their surface properties, allowing a fraction of the population to preadapt to environmental changes, is by varying protein expression through programmed genomic DNA rearrangements (1). Phase, antigenic, or size variation of expressed surface proteins are governed by mechanisms including transposition and DNA inversion. During transposition, a silent gene is activated by movement to an expression site where it displaces the currently expressed gene. In DNA inversion, a segment of DNA is cut, inverted, and then rejoined by a site-specific recombinase. The invertible DNA segment may contain either a promoter that directs expression of fixed structural genes or structural genes controlled by a fixed promoter. Transposition and inversion differ

in both the enzymes used and in the number of genes that can be controlled (many versus two).

Campylobacter fetus, a bacterial pathogen of ungulates and humans, is covered by a paracrystalline surface (S-) layer, composed of high molecular weight S-layer proteins (SLP) that masks most of the underlying gram-negative surface features (2). More than 300 bacterial genera have been described that possess S-layers (3). The S-layer renders *C. fetus* cells resistant to serum killing by prohibiting the binding of C3b (4), and the S-layer proteins themselves may change, permitting antigenic variation (5,6).

These S-layer proteins are encoded by 7-9 tightly clustered and partially homologous promoterless gene cassettes (7,8). Since previous studies show that *C. fetus* can express alternative S-layer proteins (4-6,9), there is only a single promoter for S-layer proteins expression present on a 6.2 kb invertible element (9), and the structural genes flanking the promoter are subject to substitution (9), both the promoter and the eight structural genes (*sapA* and its homologs) may rearrange strictly by inversion.

Campylobacter fetus is able to colonize the mucosa of the gastrointestinal and/or genitourinary tracts of mammals, birds and reptiles. Colonization of the wild-type organism lasts for years and can cause disease. Essential for this long term colonization is the ability to produce the S-layer proteins and *Campylobacter fetus* has the means to change the S-layer proteins and thus the crystal struture and the particular forms of antigenicity. This antigenic variation is required for the persistence of the organism in its environmental niche.

Campylobacter fetus accomplishes this antigenic variation by posssessing 7-9 highly homologous gene cassettes, called *sapA* homologs (*sapA*, *sapA1*, *sapA2*, etc.) which encode a different S-layer protein. Each of these homologs contains a 5' region of about 600 base pairs which is completely conserved from homolog to homolog and is necessary for binding of the S-layer protein encoded by that homolog to the lipopolysaccharide molecule anchored in the bacterial outer membrane. The remainder of the open reading frame (ORF) is different for each

homolog but semi-conserved regions exist. Wild type *C. fetus* strains are able to rearrange their chromosomal DNA so that the *sapA* homolog positioned downstream of a unique promoter is then expressed. This rearrangement occurs at a frequency of about 10^{-4} and is *recA* dependent. *RecA* is a protein encoded by *recA* and which is involved in homologous recombination and in repair of breaks of DNA strands.

C. fetus S-layer proteins (SLPs) are secreted in the absence of an N-terminal signal sequence. SLP proteins contain a signal sequence located within the C-terminous of the protein and are secreted through a type I protein secretion system encoded by the *sapCDEF* operon of four overlapping genes. Analysis of the C-termini of four *C. fetus* SLPs revealed conserved structures that are potential secretion signals. A *C. fetus sapD* mutant neither produced nor secreted SLPs. *E. coli* expressing *C. fetus sapA* and *sapCDEF* secreted SapA, indicating that the *sapCDEF* genes were sufficient for SLP secretion.

The prior art is deficient in the lack of effective means of delivering antigens for vaccination with a live vector. The present invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

In one embodiment of the present invention, there is provided a mutant *C. fetus* strain in which each of the cassettes is replaced by a heterologous antigen. The *sapA* homologs are altered by a DNA cassette inserted so that the encoded SLP represents a chimera between the native SLP and the peptide encoded by the cassette. The inserted DNA cassettes retain 3' *sapA* sequences that encode the C-terminal secretion signal sequences in order to ensure secretion of the chimeric protein. Representative examples of cassettes that can be inserted in this fashion include immunogens related to *Salmonella*, *Campylobacter jejuni*, *E. coli* 0157:H7, human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) as well as other enteric, venereal, or respiratory pathogens of humans, cattle, sheep, poultry, horses, swine, and reptiles. In this embodiment of the

present invention, this strain can be used to immunize a host to develop mucosal and systemic immune responses to each of the immunogens.

In another embodiment of the present invention, there is provided a mutant *C. fetus* strain in which all but one of the cassettes are replaced by a heterologous antigen. In this embodiment, one of the cassettes (e.g., *sapA2*) remains in its native configuration and the others are mutagenized. The advantage of this construction is that it also can induce immunity to *C. fetus* based on the single full-length SLP produced.

In another embodiment of the present invention, there is provided a mutant *C. fetus* strain in which *recA* is mutagenized. When the RecA protein is not produced, the DNA rearrangements permitting *sapA* antigenic variation can not occur at any detectable frequency. Thus, the *C. fetus* strain can only produce one of the SLPs encoded by one *sapA* homolog. This strain can be used, therefore, to colonize the host briefly, i.e., until protective immunity has developed to that homolog. Subsequently, the host immune response eliminates the organism. Thus, this mutant would provide effective immunity against subsequent *C. fetus* infection and is useful for vaccination of ungulates (including sheep, cattle and horses) in which infectious abortion and/or infertility can occur after the wild-type infection.

In another embodiment of the present invention, there is provided a *C. fetus* strain in which *recA* is mutagenized and the expressed *sapA* homolog is a chimera involving a heterologous peptide. In this embodiment, a mutagenized *sapA* homolog expresses a chimeric protein including a heterologous antigen. The strain is then passaged *in vitro* so that the chimeric homolog is in the expression position and then a *recA* mutation is made. This strain now essentially expresses only the chimeric protein thus providing a means to immunize a host to that antigen. The duration of colonization in the host is brief and this attenuated *C. fetus* strain allows safe immunization for the selected antigen.

In yet another embodiment of the present invention, there is provided a mixed mutant *C. fetus* strains each including a *sapA* chimera which is also a *recA* mutant. In this embodiment,

mutants are constructed in which a single *sapA* homolog is mutagenized to encode a different chimeric protein representing a different heterologous antigen. Each mutant is also RecA-deficient due to mutation in *recA*. A host is inoculated with a mixture of two or more of these strains to provide immunization to the requisite antigens. Each strain is short-lived in the immunized host.

In another embodiment of the present invention there is provided a strain of *E. coli* carrying plasmids encoding the *sapCDEF* proteins that permits the secretion of chimeric proteins containing the SapA C-terminal secretion signal. The secreted protein is encoded by an altered *sapA* homolog that has been engineered so that the 5' section of the homolog-specific region is replaced by a DNA cassette encoding a heterologous antigen. Since the chimeric protein would be secreted by *E. coli* into the culture medium, this system provides a method for obtaining a large amount of the chimeric protein in a relatively pure form.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figure 1 shows the schematic representation of serial experiments to utilize *km*- and *cm*-cassettes to examine S-layer proteins gene rearrangement (Figure 1A). Introduction of *cm* into strain 23D by marker rescue led to insertion into *sapA* to create

23D:AC100. This strain was selected on chloramphenicol-containing medium, and as expected was chloramphenicol (C) resistant, and was S^- (no S layer) and not resistant to serum (S) and kanamycin (K), as previously reported (9). Incubation of 23D:AC100 with normal human serum (NHS) selected for survivors (at a frequency of 1 x 10^{-4} (AC200 series) that were S^+ (S-layer present) and serum-resistant but sensitive to kanamycin and chloramphenicol. Strain 23D:AC200, expressing *sap*A2, was further mutagenized by introduction of *km* into *sap*A2 to create the 23D:ACA2K100 series. These strains were S^- and serum- and chloramphenicol- sensitive but kanamycin-resistant. Incubation with NHS selected for survivors (at a frequency of 1 x 10^{-4}) that were S^+ and serum-resistant but kanamycin- and chloramphenicol-sensitive (ACA2K400 series). The reciprocal relationship between antibiotic- and serum-resistance suggests that only a single promoter for S-layer protein gene cassettes is present. All ACA2K400 series strains must have an S-layer protein gene cassette other than *sap*A or *sap*A2 positioned downstream of the single S-layer protein promoter. The predicted genotypes are depicted. P, *sap*A promoter; bent arrow, location and direction of transcription of S-layer protein gene cassette; solid inverted triangles, antibiotic resistance gene insertion; NHS, normal human serum. Figure 1B shows the immunoblot of *C. fetus* strain 23D and selected mutants into which *cm* or *cm* and *km* were inserted into S-layer protein gene cassettes. As expected, all the serum-resistant strains expressed S-layer proteins (of 97 or 127 kDa) recognized by antiserum to conserved *C. fetus* S-layer proteins determinants whereas there was no expression for strains maintained on either antibiotic.

Figure 2 shows the Southern hybridization of *Hinc*II (Figure 2A) or *Pst*I (Figure 2B) digestions of chromosomal DNA from *C. fetus* 23D and ACA2K series mutants using probes to *km*, *cm*, the promoter region, the *sap*A-specific 3' region, the *sap*A1-middle region, or the *sapA*1-specific 3' region. Each probe hybridized to a single fragment regardless of the phenotype of the *C. fetus* strain. Figure 2C shows the mapping of S-layer protein gene cassette arrangement by PCR. PCRs were performed with template chromosomal DNA from strains 23D and ACA2K mutants

using *sap*A-specific 3' region forward (*sap*A) and *km* reverse (*km*) primers (left 4 lanes), *sap*A1-specific 3' region forward (*sap*A1) and *km* primers (middle 4 lanes), or *sap*A and *sap*A1-specific 3' region reverse (*sap*A1) primers (right 4 lanes). Figure 2D shows the cumulative restriction maps of the 4 strains presented in Figure 2A-2C. The location of the probes as indicated from the hybridizations is shown under the map for each strain. *sap*Ax represents an uncharacterized S-layer protein gene cassette; arrows represent direction of transcription; solid lines represent expressed genes, dashed lines represent silent genes; P over bent arrow represents the *sap*A promoter; the heavy line represents the 6.2 kb invertible promoter-containing element, flanked by opposing S-layer protein gene cassettes. The asterisks represent the palindromic putative recombinase recognition sites (TTAAGGAaTCCTTAA) present in the 5' conserved region of each S-layer protein gene cassette (7), and restriction sites are indicated: H, *Hinc*II; N, *Nde*I; P, *Pst*I.

Figure 3 shows the proposed model of molecular events involved in S-layer protein gene cassette rearrangement by DNA inversion. DNA inversion between two oppositely oriented cassettes follows DNA strand exchange at the putative recombinase target site (asterisk) found upstream of each S-layer protein gene cassette within the 5' conserved region (small grey box) (8). Patterned boxes represent variable regions of S-layer proteins gene cassettes. A 6.2kb intervening segment is topologically reversed leading to ordered rearrangement of the S-layer protein gene cassettes. Inversion of DNA segments containing the promoter ('P' over bent arrow) permits expression of alternate S-layer protein gene cassettes (mRNA, arrow). Illustrated are inversion of the 6.2 kb promoter-containing element alone (left), the 6.2 kb element and one (middle) or two (right) S-layer protein gene cassette ORFs and the resultant genotypes. Each of these genotypes has been observed (Figure 2D).

Figure 4 shows a schematic representation of the sapA invertible region, showing the sapCDEF genes, the locations of the divergent sapA and putative sapCDEF promoters (bent arrows), and the clones (pIR15, pIR13, pIR12, and pIR20) from

which the invertible region sequence was determined. The hatched areas represent the ca. 600 bp conserved regions at the 5' ends of sapA homologs flanking the invertible region (designated here as sapAx and sapAy), at which recombination occurs as the basis of sapA homolog rearrangements using S-layer protein antigenic variation.

Figure 5 shows a phylogram generated by parsimony analysis, demonstrating the relatedness of ABC transport proteins from type 1 secretion systems. The percent of amino acid similarity (%Sim) and identity (%ID) with C. fetus SapD is shown at the right. The bold numbers adjacent to the phylogram branches indicate the percentage of 1000 bootstrap replicates supporting the clustering of those branches. Branches without bootstrap values were clustered in less than 50% of bootstrap replicates.

Figure 6 shows an immunoblot detected with antiserum to SapA demonstrating the secretion of SapA from *E. coli* expressing *C. fetus* sapCEEF. One mg of whole cell proteins (lanes 1 and 2), or the amount of TCA-precipitate proteins present in 250 ml of culture supernatant (lanes 3 and 4) were analyzed. Lanes 1 and 3, C600 (pAMP1+pBCGYC1); and lanes 2 and 4, C600 (pIR100+pBCGYC1).

Figure 7 shows several models of chimeric sap homologs for the use of *C. fetus* as a live vector for antigen delivery to the mucosal immune system. The left large gray box represents the N-terminal domain required for binding of the protein to the cell surface, the right sided smaller gray box represents the C-terminal domain required for secretion of the protein. The middle region shows the native sap homolog, and then in other examples, antigens related to influenza, HIV, Shigella, a model B-subunit of a toxin, or a model A subunit of a toxin. In the chimeras without the left sided gray box, the protein would be secreted without binding to the C. fetus cell surface. RecA at right refers to possible RecA status of the host *C. fetus* strain. If the strain is $RecA^-$ then it would only express a single cassette on its surface, but if $RecA^+$ (wild type), it would be able to switch the chimera it expressed.

DETAILED DESCRIPTION OF THE INVENTION

The following abbreviations may be used herein: S-layer protein, surface layer protein; *km*, kanamycin-resistance gene; *cm*, chloramphenicol-resistance gene; NHS, normal human serum.

In the present invention, programmed gene rearrangements are employed by a variety of microorganisms, including viruses, prokaryotes, and simple eukaryotes, to control gene expression. In most instances in which organisms mediate host evasion by large families of homologous gene cassettes, the mechanism of variation is not thought to involve DNA inversion. *Campylobacter fetus*, a pathogenic gram-negative bacterium, reassorts a single promoter, controlling S-layer (surface) protein expression, and one or more complete open reading frames strictly by DNA inversion. Rearrangements are independent of the distance between sites of inversion. These rearrangements permit variation in protein expression from the large S-layer protein gene family and suggest an expanding paradigm of programmed DNA rearrangements among microorganisms. Furthermore, S-layer proteins were secreted from *C. fetus* via a type I protein secretion system encoded by the *sapCDEF* operon. Secretory signal sequences for the SapA family of secreted proteins was localized to the carboxy-terminus of these proteins.

The present invention is directed to a mutant *C. fetus* strain useful for vaccinating an animal to *Campylobacter fetus*, wherein said strain is mutated to contain a DNA cassette encoding a heterologous protein. In one embodiment of the mutant *C. fetus* strain, a *sapA* homolog is altered. In one embodiment of the mutant *C. fetus* strain, the heterologous protein is a S-layer protein. Preferably, the encoded S-layer protein represents a chimera between the native S-layer protein and the peptide encoded by the cassette. Preferably, the cassette is selected from the group consisting of *Salmonella*, *Shigella*, *Campylobacter jejuni*, *E. coli* 0157:H7, human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) and other animal pathogens. In one embodiment of the mutant *C. fetus* strain, the cassette contains a 5' binding region and 3'

secretion signal region and the protein is inserted between said binding region and said signal region. In another embodiment of the mutant *C. fetus* strain, the cassette contains a 3' secretion signal but has no binding region. Preferably, the protein is an antigen or a therapeutic agent.

The present invention is also directed to a method of immunizing a host to develop mucosal and systemic immune responses to an immunogen, comprising the step of administering to said host a pharmacologically effective dose of the strain described herein.

The present invention is also directed to a mutant *C. fetus* strain, wherein *recA* is mutated so that no functional RecA protein is produced, the DNA rearrangements permitting *sapA* antigenic variation occur at a very low frequency and wherein said *C. fetus* strain can only produce one of the S-layer proteins encoded by one *sapA* homolog. In one embodiment, this strain contains a *sapA* homolog expressing a chimeric protein including a heterologous antigen.

The present invention is also directed to a mixture of mutant *C. fetus* strains, wherein each strain includes a *sapA* chimera which is also a *recA* mutant, wherein a single *sapA* homolog is mutated to encode a different chimeric protein representing a different heterologous antigen and each mutant is also RecA-deficient due to mutation in *recA*. The present invention is also directed to a method of immunizing a host to develop mucosal and systemic immune responses to an immunogen, comprising the step of administering to said host a pharmacologically effective dose of these strains.

The present invention is also directed to a strain of bacteria modified to express *SapCDEF* genes Preferably, the strain is *Escherichia coli*. In one embodiment of this strain, a heterologous protein is expressed as a chimeric protein composed of sequences of heterologous origin, sequences that direct the secretion of said chimeric protein to the cell surface and sequences that direct the binding of the secreted chimeric protein to the lipopolysaccharides of the bacterial cell surface via the sapCDEF directed type 1 secretory system. The present invention is also directed to a method of immunizing a host to generate immune responses to an

immunogen,. comprising the step of administering to said host a pharmacologically effective dose of this strain.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

Bacterial strains and culture conditions

Wild-type S^+ (possessing an S-layer) *C. fetus* strain 23D and spontaneous S^- (no S-layer present) mutant strain 23B have been extensively characterized (4,5,12,13). Other *C. fetus* strains used were defined mutants derived from strain 23D, as described below. Stock cultures were stored and grown as described elsewhere (8). PVNT media containing 7 U/ml polymyxin B, 10 µg/ml vancomycin, 50 µg/ml naladixic acid, 10 µg/ml trimethoprim lactate, were supplemented for kanamycin-resistant strains or chloramphenicol-resistant strains, with 30 µg/ml kanamycin or 15 µg/ml chloramphenicol, respectively. *E. coli* strains used, including DH5α, HB101 and XL1-Blue (Stratagene, La Jolla, Calif.), were grown in L broth or on L plates (14).

EXAMPLE 2

Chemicals and enzymes

Isopropyl-β-D-thiogalactopyranoside (IPTG) (50 µg/ml) and 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal) (28 µg/ml) were purchased from Jersey Lab Supply (Livingston, NJ.). Restriction enzymes, T4 DNA ligase, Taq polymerase and *E. coli* DNA polymerase large (Klenow) fragment were from Promega and U.S. Biochemical Corp. (Cleveland, OH.). Antibiotics were from Sigma Chemical Co. (St. Louis, MO.), and [α-^{32}P]dATP (650 mCi/mmol) was from ICN Radiochemicals (Irvine, Calif.).

EXAMPLE 3

Genetic techniques

Chromosomal DNA was prepared from 48 hour plate cultures, as described (9). Plasmids were isolated by the

procedure of Birnboim and Doly (15). All other standard molecular genetic techniques were done, as described (14).

EXAMPLE 4

Construction of mutant *C. fetus* strains

Mutant *C. fetus* strains were created by mobilization of donor pKO500 or pKO505 plasmid constructs by conjugal mating as described elsewhere (9) with sequential selection (as shown in Figure 1) on media containing 30 µg/ml kanamycin or 15 µg/ml chloramphenicol, or in the presence of 10% normal human serum (NHS), as described (16). pKO500: suicide hybrid plasmid with the *sap*A open reading frame disrupted with a chloramphenicol-resistance gene (*cm*) (17) located 127 base pairs into the open reading frame. pKO505: suicide hybrid plasmid with the *sap*A 2 open reading frame disrupted with a promoterless kanamycin-resistance gene (18) located 127 base pairs into the open reading frame .

EXAMPLE 5

Bactericidal assays

To determine the susceptibilities of the mutant strains to the bactericidal activity of normal human serum, 10-fold serially-diluted cultures (starting from a single colony) were incubated at 37^{0}C for 60 minutes in the presence of 10% pooled normal human serum or 10% heat-inactivated normal human serum, as described (9, 16). Wild-type S^+ strain 23D and spontaneous S^- mutant strain 23B were the serum-resistant and serum-sensitive controls, respectively (9). Cultures then were plated to media containing chloramphenicol, kanamycin, or no antibiotic selection (Figure 1A), and following incubation, bacterial colonies were enumerated. Survival rates were determined as the ratio of colony forming units (cfu)/ml in the presence of normal human serum or heat-inactivated human serum, or a similar ratio of cfu/ml in the presence or absence of the selective antibiotic.

EXAMPLE 6

Production of antiserum to *C. fetus* S-layer proteins

Antiserum to the 97 kDa S-layer proteins of type A strain 82-40LP was raised in adult New Zealand White female

rabbits and shows broad recognition of *C. fetus* S-layer proteins as described (5). To analyze wild-type and transconjugant *C. fetus* strains for S-layer proteins expression, cells were harvested from plates, lysed in sample loading buffer and examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting was performed as detailed (5,13).

EXAMPLE 7

Southern hybridizations and probes

C. fetus chromosomal DNA was digested with *Hinc*II and processed exactly as described (12). Probes included the gel-purified PCR products specific for the *sap*A promoter region (9), *km*, *cm*, 3' *sap*A region (1649 to 2760 bp), middle *sap*A1 region (620 to 1381 bp) and 3' *sap*A1-region (1381 to 2763 bp). Probes were ^{32}P-radiolabeled by primer extension with random hexameric oligonucleotides (19). Polymerase chain reactions were performed as described (9).

EXAMPLE 8

Polymerase chain reaction

Polymerase chain reactions were performed as described (9). Amplification of large PCR products was accomplished by denaturing for only 5 seconds and extending at 68^{0}C for longer periods, typically 4-7 minutes. Primers used include:

km F, 5'-TGTAGAAAAGAGGAAGGAAA-3' (SEQ ID No: 1);

km R; 5'-CTAAA ACAATTCATCCAGTA-3' (SEQ ID No: 2);

cm F, 5'-AGTGGATAGATTTATGATATAGTG-3' (SEQ ID No: 3);

cm R, 5'-T TTATTTATTCAGCAAGTCTTG-3' (SEQ ID No: 4);

*sap*A F middle, 5'-CATCTCTACAGCAGCAAAAG-3' (SEQ ID No: 5);

*sap*A F 5'-GCGGAGATAATGTTGTAGTTGAT G-3' (SEQ ID No: 6);

*sap*A R, 5'-AACTTTAAGAT CTAGCGTACC-3' (SEQ ID No: 7);

*sap*A1 F middle, 5'-AGGGTACTGATTTAGACGATA-3' (SEQ ID No: 8);

*sap*A1 F 3', 5'-GCTGGATTTACAGGAGATTTAACC-3' (SEQ ID No: 9);

*sap*A1 R 3' #1, 5'-GTTACTGGTATCAATAA CAACATAAGT-3' (SEQ ID No: 10);

*sap*A1 R 3' #2, 5'-CTACGTAATCATACTGCTACC-3' (SEQ ID No: 11).

EXAMPLE 9

Construction and phenotypic analysis of mutant strains

To show that both the *sap*A promoter and the complete structural genes can be mobilized by DNA inversion, mutant strains were created in which both S-layer protein-encoding gene cassettes bracketing the invertible *sap*A promoter were disrupted (Figure 1A). First, insertion of a chloramphenicol-resistance cassette (*cm*) into the open reading frame (ORF) of the expressed S-layer gene cassette (*sap*A) ablated SapA expression (Figure 1B) and rendered the organism (23D:AC100) serum-sensitive. Using the ability of S^+ (but not S^-) *C. fetus* strains to survive incubation in normal human serum (4, 13, 20-22), mutants (9) expressing the *sap*A2 cassette were then selected for promoter inversion and identified strain 23D:AC200 (Figure 1). Next, 23D:AC200 was mutagenized by insertion of the kanamycin-resistance cassette (*km*) to ablate SapA2 expression, creating 23D:ACA2K101 (Figures 1 and 2D). Incubation of this dually disrupted strain in serum selected for serum-resistant survivors. These were found at a frequency of 1×10^{-4}, and as expected, expressed S-layer proteins of 97 kDa or 127 kDa (Figure 1B) and were antibiotic-sensitive. Immunoblot of 26 serum survivors demonstrated that 16 (62%) expressed a 97 kDa (e.g., 23D:ACA2K400) and 10 (38%) expressed a 127 kDa (e.g., 23D:ACA2K450) S-layer proteins. These results suggested that in each of the survivors there had been exchange of at least one of the bracketing cassettes. None of the mutant strains maintained on antibiotic-containing media produced an S-layer proteins band, as revealed by immunoblot, (Figure 1b), and this shows that expression of *cm* or *km* is dependent on the single *sap*A promoter. These experiments provided a group of well-defined strains with

which to examine the genotypic events associated with the observed phenotypic variations.

EXAMPLE 10

Phenotypic variation is associated with nested DNA inversions

To investigate the nature of the recombination event that allowed the single *sap*A promoter to express a native S-layer gene cassette in the dually disrupted strains, using Southern analyses, these organisms were compared with wild-type *C. fetus* strain 23D and the S⁻ parental mutant 23D:ACA2K101. The known positions of *Hin*cII (Figure 2A), *Pst*I (Figure 2B), and *Nde*I (data not shown) sites located in *sap*A, *sap*A1 and *sap*A2 (8,9,23,24) were used to predict the size of the fragments hybridizing with appropriate probes. The promoter region, the *sapA*-3' region, the *sapA*1 middle region and the *sap*A1-3' region probes hybridized to 8.5 kb, 4.8 kb, 4.8 kb and 4.3 kb *Hin*cII fragments, respectively, in wild-type strain 23D (Figure 2A). Based on these and previous Southern hybridization and PCR data (9), the location of these genes relative to one another in wild-type strain 23D was defined (Figure 2D, top line).

An identical hybridization pattern was observed for mutant 23D:ACA2K101 with the exception that the *km* and *cm* probes hybridized to a 10.4 kb fragment and that the promoter region probe also hybridized to a 10.4 kb fragment, (Figure 2D, line two). These results are entirely consistent with the introduction of the *km* (0.8 kb) and *cm* (1.1 kb) markers flanking the promoter. For the mutant strains ACA2K400 and ACA2K450, changes in phenotype were clearly associated with change in probe co-hybridization (Figure 2A). In mutant strain 23D:ACA2K400, the *km* probe hybridized to the same 5.1 kb fragment as the *sap*A-3' region probe and in strain 23D:ACA2K450 to the same 4.0 kb fragment as the *sap*A1-3' region probe (Figure 2A), whereas the promoter region probe hybridized to fragments larger than 9 kb. For each of the mutant strains, the *cm* and promoter region probes hybridized to fragments of identical sizes (Figure 2A). These results demonstrate that the open reading frames had inverted (Figure 2D), and reflect the differing locations

of each of the S-layer protein gene cassettes in relation to the *km* marker. The total size of non-overlapping hybridizing fragments remained constant among the strains (17.6 kb in wild-type and, reflecting addition of the two antibiotic resistance cassettes, 19.5 kb in all mutant strains) indicating that recombination did not involve net duplication or deletion of DNA (Figure 2d).

Following *Pst*I digestion, as expected, the *km* probe hybridized to the same 7.3 kb, 6.5 kb, and 5.9 kb fragments as the promoter region probe, the *sap*A-3' region probe and the *sap*A1-3' region probe in strains ACA2K101, ACA2K400 and ACA2K450, respectively (Figure 2B). Despite the genotypic differences among the mutant strains, the constancy of the 3.1 kb fragment hybridizing with the *cm* probe (Figure 2B) was consistent with the position of the marker downstream of the promoterless end of the 6.2 kb invertible element (Figure 2D). Hybridization of the promoter region probe to 5.5 kb, 4.9 kb and 5.3 kb fragments in strain 23D and mutants ACA2K400 and ACA2K450, respectively, also reaffirms the model. The *sap*A-3' region probe hybridized with a 4.1 kb fragment in strains 23D, and mutants ACA2K101 and ACA2K450, but not ACA2K400 (Figure 2B), consistent with the locations of *Pst*I sites within *sap*A and *sap*A1, and the tandem relationship between these two cassettes. Similarly, the *sap*A1-3' region probe hybridized with a 4.2 kb fragment in strains 23D, ACA2K101 and ACA2K400, but not ACA2K450 (Figure 2B), consistent with the tandem relationship between *sap*A1 and the downstream *sap*Ax in the first three strains. The observed sizes of each of the hybridizing fragments and their co-hybridization patterns in *Pst*I (Figure 2B) or *Nde*I-digested (not shown) genomic DNA using similar probes were completely consistent with the known restriction sites (8,9,23,24) as depicted in Figure 2D.

PCR analyses provided independent confirmation for open reading frame inversion. The 3' *sap*A-specific forward primer and a *km* reverse primer yielded a product for strain 23D:ACA2K400 (Figure 2C), indicating that *sap*A had inverted and was located immediately upstream of *km*. Similarly, for strain 23D:ACA2K450 the data (Figure 2C) indicate that *sap*A1 had inverted and was located immediately upstream of *km*. PCR analyses using the 3' *sap*A-specific forward primer and 3' *sap*A1-

specific reverse primer yielded a 4.3 kb product for all strains except for 23D:ACA2K400 (Figure 2C, rightmost 4 lanes), indicating that the tandem relationship of *sap*A and *sap*A1 was lost in strain ACA2K400. The data indicate that for mutant strains 23D:ACA2K400 and 23D:ACA2K450, *sap*A, or both *sap*A and *sap*A1, respectively, have inverted in relationship to *sap*A 2 (Figure 2D, lines 2-4).

EXAMPLE 11

DNA inversion events occur independent of the distance between recombination sites

The frequency of the DNA inversion events was determined, involving promoter alone, or promoter and one or more of the gene cassettes. The mutant strains (TABLE 1) provided easily definable phenotypes with which to assess the inversion events. These were measured by conducting experiments in which the strains were examined for resistance to a selection that would be lethal (exposure to kanamycin, chloramphenicol, or serum) unless defined inversions allowing expression of genes to overcome the exposure had occurred. Events involving inversion of the promoter-containing element alone, or together with one or two open reading frames, occurred at nearly equal frequency (~1 to 2.6 X 10^{-4}) (TABLE 1). These data imply that inversions involving two adjacent open reading frames occurred in a single event and did not result from two or more sequential inversion events. Recombination occurs at either homologous or palindromic DNA sequences (9). The distance-independence between sites suggests that inversion occurs by a random collision model, as proposed (25).

TABLE 1

C. fetus survival after serum or antibiotic selection

Strain	Relevant Phenotype: Susceptibility to Chlor.	Kana.	Serum	Immunoblot presence of S layer; kDa	Selection*	No. Exp.
23D:AC100	R	S	S	---	Serum	7
23D:AC200	S	S	R	127	Chloram.	6
23D:ACA2K101	S	R	S	---	Chloram.	12
23D:ACA2K101	S	R	S	---	Serum	8
23D:ACA2K200	R	S	S	---	Kanamycin	3
23D:ACA2K400	S	S	R	97	Kanamycin	6
23D:ACA2K400	S	S	R	97	Chloram.	6
23D:ACA2K450	S	S	R	127	Kanamycin	5
23D:ACA2K450	S	S	R	127	Chloram.	6

S: sensitive; R: resistant; Chloram.: chloramphenicol *Selection by plating cells with 15 µg/ml chloramphenicol or 30 µg/ml kanamycin, or incubating cells in 10% NHS

EXAMPLE 12

Cloning of the invertible region

Since bacterial genes involved in similar functions are often clustered, the 6.2 kb invertible region between two *sapA* homologs was characterized. The 6.2 kb fragment was first amplified by PCR, and this product was subcloned into pAMP1 to yield the plasmid pIR100. Next, this subcloned fragment was used as a probe to isolate a series of overlapping plasmid clones derived from a *C. fetus* 23D genomic library constructed in λZAPII. Four of these, designated pIR15, pIR13, pIR12, and pIR20 represented the majority of the invertible region and were subjected to DNA sequence analysis. In order to determine the sequence of the small sequence gap between pIR13 and pIR12, a DNA segment was amplified from *C. fetus* 23D genomic DNA by PCR using appropriate primers and subcloned into pT7Blue. To avoid the problem of PCR-induced errors, three independent subclones were sequenced and in each case the sequence was identical.

EXAMPLE 13

Analysis of invertible region features

The DNA sequence of the 6229 bp invertible region from strain 23D was predicted to contain four open reading frames, which were designated *sapCDEF* (Figure 4). The *sapC* gene began 596 bp from the initiation codon of the oppositely-oriented upstream *sapA* homolog. The *sapC* open reading frame was 1035 bp in length and was immediately followed by the *sapD*, *sapE*, and *sapF* open reading frames, which were 1752, 1284, and 1302 bp, respectively. Each gene in this cluster had a typical ribosome binding site, and overlapped the preceding gene, by 14 bp (*sapC/D*), 1 bp (*sapD/E*), and 11 bp (*sapE/F*). The *sapF* gene ended 287 bp upstream of the *sapA* homolog located downstream. The 74 bp preceding the ATG codons initiating translation of the *sapA* homologs flanking *sapCDEF* were identical to each other (Figure 4). These conserved segments have previously been noted upstream of the three characterized *sapA* homologs, and may play a role in

the inversion of this DNA segment. As a potential component of this recombination mechanism, sequences resembling χ sites (RecBCD recognition) were present at positions 31-38 and 6192-6199. Several potential σ^{70}-like promoters were noted 44-243 bp upstream of *sapC*. These putative *sapCDEF* promoters were oriented in the opposite direction to the *sapA* promoter, with the two -35 regions separated by 200-380 bp. Due to the overlapping nature of the *sapCDEF* genes and the lack of other putative promoters, it is likely that they are co-transcribed.

EXAMPLE 14

Similarities of SapCDEF to other proteins

The *sapC* open reading frame predicted a protein product of 344 amino acids (39.7 kDa) that had no significant similarities in the non-redundant database maintained by NCBI. In contrast, the products of the *sapDEF* genes had high similarity to proteins encoding type I secretion systems. SapD (584 amino acids, 64.0 kDa) was related to the ABC family of transporters, especially those that are involved in the translocation by type I secretion systems of proteases, lipases, hemolysins, and leukotoxins across the envelopes of gram-negative bacterial pathogens. The degree of similarity between SapD and these proteins was between 68% similarity (with *Pseudomonas aeruginosa* AprD) and 50% similarity (*Actinobacillus actinomycetemcomitans* LktB). In addition, SapD contained two motifs found in such proteins, an ATP/GTP binding site (GPSAAGKS; Walker Box A, amino acid residues 365-377) and a peptide (LSGGQRQRVALA, amino acid residues 468-479) that is a signature sequence for ABC transporters. The SapE protein (428 aa, 47.9 kDa) was similar to the MFP proteins of type I transporters, typified by *P. aeruginosa* AprE (52% similar) and *E. coli* HlyD (49% similar). SapF (434 amino acids, 49.4 kDa) was related to the outer membrane component of type I systems, such as *P. aeruginosa* AprF (45% similar), *Erwinia chrysanthemi* PrtE (41% similar), and *E. coli* TolC (47% similar). Parsimony and bootstrap analyses supported the classification of the *C. fetus*

transport proteins on phylogenetic branches distinct from other type I secretion apparatuses (Figure 5).

EXAMPLE 15

Construction of a sapD mutant

To examine whether genes in the *C. fetus* invertible region encoded proteins responsible for SLP secretion, a derivative of type A strain 23D containing an insertional mutation in *sapD* was constructed. To generate a *sapD* mutation, an *aphA* cassette was inserted into a unique BglII site within *sapD* and transformants were selected on plates containing kanamycin. This new clone was designated pIR131. The *sapD* insert containing the resistance fragment was subcloned into suicide vector pILL570 to yield pILL131, which was transformed into *E. coli* S17-1. Transformants were selected from trimethoprim/kanamycin plates and verification of pILL131 was made by digestion with *HindIII*. The mob$^+$ *E. coli* strain S17-1 containing IncP DNA transfer functions and pILL131 was used as the conjugation donor, and *C. fetus* wild-type strain 23D was the recipient. Approximately five thousand transconjugants were recovered (frequency of 4 x 10^{-6} transconjugants per recipient). Chromosomal DNA from one of these strains was extracted and digested with *NdeI* for Southern analysis, using hybridization probes for *sapD*, *aphA*, and pILL570. The *sapD* probe hybridized with a 4.2 kb *NdeI* fragment in wild-type strain 23D, exactly as predicted from the DNA sequence of the invertible region. The size of the *NdeI* fragment in strain 97-205 hybridizing to the *sapD* probe was 5.6 kb indicating the incorporation of the 1.4 kb *aphA* cassette in *sapD*, as expected. The hybridization of the *aphA* probe with a fragment of 5.6 kb in 97-205 but not in 23D was consistent with this observation. A pILL570 probe did not hybridize with DNA from 97-205, indicating that 97-205 was derived from a double crossover event in which only the mutagenized *sapD* allele was incorporated into the chromosome. PCR experiments using *sapD*- and *aphA*-specific primers confirmed the Southern hybridization data. These results indicate that a strain containing an insertional mutation in *sapD* has been successfully constructed.

EXAMPLE 16

Properties of a *C. fetus* sapD mutant

Whether the *C. fetus sapD* mutant strain 97-205 was able to export S-layer proteins and to assemble a functional S-layer on its surface was next determined. First, the ability of the wild-type and *sapD* mutant strain to resist complement-mediated killing, a phenotype consistent with the presence of the S-layer was examined. Suspensions of cells were exposed either to normal human serum or to heated-inactivated normal human serum, and the extent of complement-mediated killing was determined. As expected, the S^+ strain 23D was completely resistant to killing (0.31 $\log_{10}$ kill). In contrast, as expected, there was approximately three $\log_{10}$ killing of strain 23B (3.34 $\log_{10}$ kill), that is unable to express an S-layer. Results for strain 97-205, the *sapD* mutant (3.04 $\log_{10}$ kill), were nearly identical to that for 23B, and are consistent with the absence of a functional S-layer on the surface of *sapD* mutant strain 97-205.

To understand the basis for the serum-susceptibility of the *sapD* mutant, immunoblots were performed to detect the presence of SLPs on the cell surface. S-layer proteins can readily be removed from the surface of S^+ cells by washes with distilled water. Therefore, water washes and whole-cell proteins of wild-type and *sapD* mutant cells were analyzed by immunoblot with polyclonal antiserum against SLPs. In the wild-type strain, S-layer proteins were detected both in whole-cell samples and water washes, as expected for cells expressing a functional S-layer, whereas no S-layer proteins were detected for S^- strain 23B. In the *sapD* mutant, however, S-layer proteins were not detected either extracellularly or in whole-cell samples. Thus, disruption of *sapD* by the insertion of an antibiotic-resistance cassette had the effect of eliminating S-layer proteins expression altogether, as well as their secretion. Primer extension analysis indicated approximately wild-type levels of *sapA* mRNA in the *sapD* mutant 97-205, indicating that the effect of the *sapD* mutation on the inability to detect SLPs in the cytoplasm was not due to a regulatory effect on S-layer protein gene transcription.

EXAMPLE 17

Secretion of SapA from *E. coli* expressing sapCDEF

To determine whether *sapCDEF* permits the secretion of SapA, the ability of *E. coli* strains carrying pIR100 to mediate the specific secretion of *C. fetus* SapA was tested. *E. coli* C600 cells were isolated that had been transformed with pBGYC1, a pACYC184-derived plasmid containing *C. fetus sapA*, and either pIR100 (*sapCDEF*) or pAMP1 alone (vector control). SapA secretion was assayed using immunoblots to indicate the appearance of SapA in filtered, trichloroacetic acid (TCA)-precipitated culture supernatants prepared from these strains. As expected, both *E. coli* strains produced SapA (Figure 6). Secretion of SapA was detected only in supernatants from *E. coli* cells expressing *sapCDEF* (Figure 6). These results indicate that the *C. fetus sapCDEF* genes were sufficient to allow *E. coli* to secrete SapA.

EXAMPLE 18

Conserved features of S-layer protein C-termini

Proteins that are transported by type I secretion systems do not have the N-terminal signal peptides that are conserved in proteins exported by type II systems. Type I secretory proteins rely upon signals that are located at their extreme C-termini. However, these signals tend to have little primary sequence homology, and their exact structures have been difficult to elucidate. In an attempt to define candidate sequences/structures for C-terminal secretion signals in bacterial SLPs, the C-terminal 70 amino acids of four *C. fetus* SLPs for which the sequences are known (SapA, SapA1, SapA2, and SapB) were aligned.

Several conserved peptides were evident. The sequence GDGS(T/G) was present in three of the four S-layer proteins, with SapA2 possessing a slightly different version (skGST). Similarly, the peptide GxTYVV(V/I)D was present in three of the four SLPs, with the corresponding sequence (GxTYVVda) of SapA2 again being slightly more divergent. Several other conserved amino acid residues also were apparent. The DVIV motifs implicated in protease secretion were not

present per se at the extreme C-terminus of any of the S-layer proteins, although similar sequences (DGSVI) were found at the C-termini of the SapA and SapB proteins. Similar to type I-secreted toxins and related exoproteins, the S-layer proteins had 1-4 hydroxylated residues (S or T) within the C-terminal ten amino acids.

To investigate whether the C-terminal domains of these S-layer proteins also could form similar secondary structures, each of these peptides was analyzed using the programs of Garnier et al. The predicted C-terminal secondary structures (α-helix, β-sheet, amphipathic peptides, and turn-forming residues) were then superimposed on the alignment of the 4 C-terminal peptides. Three of these peptides (SapA, SapA1, and SapB) consisted of segments predicted to form sheet-sheet-helix-sheet, with each of these domains separated by turn-forming. The C-terminal domain of SapA2 was predicted to form a structure of helix-sheet-helix-sheet. Furthermore, the sheet-forming regions of all 4 proteins, as well as the most N-terminal helix-forming region of SapA2, were predicted to be amphipathic. Taken together, these results suggest that the C-termini of four *C. fetus* S-layer proteins contain conserved sequences and secondary structures that are candidates for secretion signals.

S-layer proteins expression in *C. fetus* is based on the single *sap*A promoter present on an invertible 6.2 kb element, that is bracketed by inverted repeats and oppositely oriented cassettes, *sap*A and *sap*A2, that are subject to substitution (9). By creation of appropriate mutants and by using selection for phenotypic properties, the present invention demonstrates that DNA rearrangement can involve inversion of this element in concert with one or more of the tandemly arrayed S-layer protein gene cassettes.

DNA inversion has been believed to involve mutually exclusive promoter or structural gene inversion. Either the promoter inverts relative to fixed structural genes (26,27), or structural genes invert downstream of a fixed promoter (28-30) permitting expression of two alternate gene copies (10,11). The system of DNA inversion in *C. fetus* is novel because it combines the features of each mechanism as both the promoter and the

structural genes are mobile, which permits the shuffling of complete genes and their ultimate expression (Figure 3). Rearrangement of the S-layer protein gene cassettes permits the organism to vary S-layer protein expression and surface antigenicity allowing for evasion of host immune responses. This inversion system differs from the *Mycoplasma pulmonis vsa* gene inversion, which rearranges only incomplete coding regions and demonstrates less sequence stability.

The present invention further indicates that inversion occurs randomly between open reading frames of opposite orientation independent of the size of the intervening DNA segment. The economy of a simple inversion system may be especially useful for *C. fetus* which has a relatively small (1.1 megabase) genome (32); the strict conservation of both coding and non-coding regions related to the *sap* homologs in type A and type B strains (7) is consistent with the importance of this efficient system. The present invention expands the paradigms of DNA rearrangement, in which large gene families of complete open reading frames can reassort by inversion so as to vary the surface protein expression of the microbe.

EXAMPLE 19

Mutant *C. fetus* strain with cassettes replaced by a heterologous antigen

Each of the *sapA* homologs is altered so that the central portion of the homolog-specific region is replaced by a DNA cassette encoding a heterologous antigen. Representative examples of cassettes that can be inserted are immunogens related to *Salmonella*, *Campylobacter jejuni*, *E. coli* 0157:H7, human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) as well as venereal, gastrointestinal, or respiratory pathogens of humans, cattle, sheep, poultry, horses, swine, and reptiles. The replacement is constructed so that the encoded S-layer protein is a tripartite chimera consisting of the N-terminal LPS-bining domain of the S-layer protein, a central region composed of the heterologous antigen, and a C-terminal segment containing the S-layer protein secretion signal. This protein would be able to be secreted from the cell by means of the C-terminal

secretion signal and into the LPS on the *C. fetus* cell surface, thereby exposing the heterologous antigen to the host immune system. Because the natural cassettes are replaced by the chimerae, one embodiment of the invention is the expectation that carriage of this organism is substantially shorter than for the wild type strain and thus, self-limiting. This strain should colonize for less than 3 months allowing the host to develop a mucosal and systemic immune response to each of the immunogens.

In another embodiment, one may use a mutant *C. fetus* strain in which all but one of the cassettes is replaced by a heterologous antigen. This embodiment is similar to Example 12 except that one of the cassettes (e.g., *sapA2*) remains in its native configuration but that the others are mutagenized to form chimerae. The advantage of this construction is that one can induce immunity to *C. fetus* based on the single full-length S-Layer protein produced.

EXAMPLE 20

Mutant *C. fetus* strain in which *recA* is mutagenized

In another embodiment of the present invention, one may mutagenize *recA* so that the RecA protein is not produced and therefore, the DNA rearrangements permitting *sapA* antigenic variation can not occur at any detectable frequency. Thus, the *C. fetus* strain can only produce one of the S-Layer proteins encoded by one *sapA* homolog. This strain should only colonize the host briefly, i.e., until protective immunity has developed to that homolog. Subsequently, the host immune response should eliminate the organism. Thus, this mutant would provide effective immunity against subsequent *C. fetus* infection and is useful for vaccination of ungulates (including sheep, cattle and horses) in which infectious abortion and/or infertility can occur after the wild-type infection.

EXAMPLE 21

C. fetus strain in which *recA* is mutagenized and the *sapA* homolog that is being expressed is a chimera involving a heterologous peptide

In this embodiment, a *sapA* homolog is mutagenized to allow expression of a chimeric protein including a heterologous antigen. The strain is then passaged in vitro so that the chimeric homolog is in the expression position and then a *recA* mutation is made. This strain now expresses only the chimeric protein which can serve as a means to immunize a host to that antigen. Since the strain can not vary and is not producing a native S-Layer protein, the duration of colonization in the host is brief (days). This attenuation of the *C. fetus* strain allows safe immunization for the selected antigen.

EXAMPLE 22

Mixed mutant *C. fetus* strains each including a *sapA* chimera which is also a *recA* mutant

In this embodiment, a series of mutants are constructed in which for each a single *sapA* homolog is mutagenized with the replacement to encode a different chimeric protein, each one representing a different heterologous antigen. Each mutant is also RecA-deficient due to mutation in *recA*. A host is inoculated with a mixture of two or more of these strains to provide immunization to the requisite antigens. Each strain is short-lived in the immunized host.

EXAMPLE 23

Mutant *C. fetus* strain that secretes a heterologous antigen

Each of the *sapA* homologs is altered so that the 5' section of the homolog-specific region is replaced by a DNA cassette encoing a heterologous antigen. Examples of the cassettes that can be inserted are immunogens related to *Salmonella, Campylobacter jejuni, E. coli* O157:H7, human immunoeficiency virus (HIV), simian immunoeficiency virus (SIV) as well as veneral, gastrointestinal, or respiratory pathogens of humans, cattle sheep, poultry, horses, swine, and reptiles. The replacement is constructed so that the encoded S-layer protein is a bipartite chimera consisting a central region composed of the heterologous antigen and a C-terminal segment containing the S-layer protein secretion signal. This protein would be able to be secreted from the cell by means of the C-terminal secretion signal thereby

exposing the heterologous antigen to the host immune system. Because the natural cassettes are replaced by the chimerae, one embodiment of the invention is the expectation that carriage of this organism is substantially shorter than for the wild type strain and thus, self-limiting. This strain should colonize for less than 3 months allowing the host to develop a mucosal and systemic immune response to each of the immunogens.

In another embodiment, one may use a mutant *C. fetus* strain in which all but one of the cassettes is replaced by a heterologous antigen. This embodiment is similar to Example 12 except that one of the cassettes (e.g., *sapA2*) remains in its native configuration but that the others are mutagenized to form chimerae. The advantage of this construction is that one can induce immunity to *C. fetus* based on the single full-length S-Layer protein produced.

The following references were cited herein:

1. Borst, P. & Greaves, D. R. (1982) *Science* **235**, 658-667.
2. Fujimoto, et al., (1991) *Infect. Immun.* **59**, 2017-2022.
3. Beveridge, T.J., Koval, S.F. (1993) Advances in paracrystalline bacterial surface layers. *Plenum Press, New York.* 1-344.
4. Blaser, et al., (1988) *J. Clin. Invest.* **81**, 1434-1444.
5. Wang, et al., (1993) *J. Bacteriol.* **175**, 4979-4984.
6. Garcia, et al., (1995) *J. Bacteriol.* **177**, 1976-1980.
7. Dworkin, et al., (1995) *J. Biol. Chem.* **270**, 15093- 15101.
8. Dworkin, et al., (1995) *J. Bacteriol.* **177**, 1734-1741.
9. Dworkin, et al., (1996) *Mol. Microbiol.* **19, 1241-1253**.
10. Glasgow, et al., (1989) in *Bacterial DNA inversion systems*, eds. Berg, et al., (ASM, Washington, D.C.), pp. 637-659.
11. van de Putte, et al., (1992) *Trends in Genetics* **8**, 457-462.
12. Tummuru, et al., (1992) *J. Bacteriol.* **174**, 5916-5922.
13. Blaser, et al., (1987) *J. Infect. Dis.* **155**, 696-705.
14. Sambrook, et al., (1989) *Molecular cloning: a laboratory manual*, Cold Spring Harbor Lab., Cold Spring Harbor, N.Y.).
15. Birnboim, et al., (1979) *Nucleic Acids Res.* **7**, 1513-1523.
16. Blaser, et al., (1985) *J. Infect. Dis.* **151**, 227-235.
17. Wang, E. & Taylor, D. E. (1990) *Gene* **94**, 23-28.
18. Trieu-Cout, et al., (1985) *EMBO J.* **4**, 3583-3587.
19. Feinberg, et al., (1983) *Anal. Biochem.* **132**, 6-13.

20. Pei, Z. & Blaser, M. J. (1990) *J. Clin. Invest.* **85,** 1036-1043.
21. Winter, et al., (1978) *Infect. Immun.* **22,** 963-971.
22. Blaser, M. J. & Pei, Z. (1993) *J. Infect. Dis.* **167,** 696-706.
23. Blaser, et al., (1990) *J. Biol. Chem.* **265,** 14529-14535.
24. Tummuru, M. K. R. & Blaser, M. J. (1993) *Proc. Natl. Acad. Sci. USA.* **90,** 7265- 7269.
25. Gellert, M. & Nash, H. (1987) *Nature (London)* **325,** 401-404.
26. Silverman, et al., (1979) *Proc. Natl. Acad. Sci. USA* **76,** 391-395.
27. Abraham, et al., (1985) *Proc. Natl. Acad. Sci. USA* **82,** 5724-5727.
28. Marrs, et al., (1988) *J. Bacteriol.* **170,** 3032-3039.
29. Iida, et al., (1982) *EMBO J.* **1,** 1445-1453.
30. Giphart-Gassler, et al., (1982) *Nature (London)* **297,** 339-342.
31. Blaser, et al., (1994) *Mol. Microbiol.* **14,** 453-462.
32. Salama, et al., (1992) *Int. J. System. Bacteriol.* **42,** 446-450.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. A mutant *C. fetus* strain useful for vaccinating an animal to *Campylobacter fetus*, wherein said strain is mutated to contain a DNA cassette encoding a heterologous protein antigen.

2. The mutant *C. fetus* strain of claim 1, wherein a *sapA* homolog is altered.

3. The mutant *C. fetus* strain of claim 1, wherein said heterologous protein is a S-layer protein.

4. The mutant *C. fetus* strain of claim 1, wherein the encoded S-layer protein represents a chimera between the native S-layer protein and the peptide encoded by the cassette.

5. The mutant *C. fetus* strain of claim 1, wherein said cassette is selected from the group consisting of *Salmonella*, *Shigella*, *Campylobacter jejuni*, *E. coli* 0157:H7, human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) and animal pathogens.

6. The mutant *C. fetus* strain of claim 1, wherein said cassette contains a 5' binding region and 3' secretion signal region and wherein said protein is inserted between said binding region and said signal region.

7. The mutant *C. fetus* strain of claim 1, wherein said cassette contains a 3' secretion signal but has no binding region.

8. The mutant *C. fetus* strain of claim 1, wherein said protein is selected from the group consisting of an antigen and a therapeutic agent.

9. A method of immunizing a host to develop mucosal and systemic immune responses to an immunogen, comprising the step of administering to said host a pharmacologically effective dose of the strain of claim 1.

10. A mutant *C. fetus* strain, wherein *recA* is mutated so that no functional RecA protein is produced, the DNA rearrangements permitting *sapA* antigenic variation occur at a very low frequency and wherein said *C. fetus* strain can only produce one of the S-layer proteins encoded by one *sapA* homolog.

11. The mutant *C. fetus* strain of claim 10, wherein said strain contains a *sapA* homolog expressing a chimeric protein including a heterologous antigen.

12. A mixture of mutant *C. fetus* strains, wherein each strain includes a *sapA* chimera which is also a *recA* mutant, wherein a single *sapA* homolog is mutated to encode a different chimeric protein representing a different heterologous antigen and each mutant is also RecA-deficient due to mutation in *recA*.

13. A method of immunizing a host to develop mucosal and systemic immune responses to an immunogen, comprising the step of administering to said host a pharmacologically effective dose of the strains of claim 12.

14. A strain of bacteria modified to express *SapCDEF* genes.

15. The strain of claim 14, wherein said bacterium is *Escherichia coli*.

16. The strain of claim 14, wherein a heterologous protein is expressed as a chimeric protein composed of sequences of heterologous origin, sequences that direct the secretion of said chimeric protein to the cell surface and sequences that direct the binding of the secreted chimeric protein to the lipopolysaccharides of the bacterial cell surface via the sapCDEF directed type 1 secretory system.

17. A method of immunizing a host to generate immune responses to an immunogen, comprising the step of administering to said host a pharmacologically effective dose of the strain of claim 14.

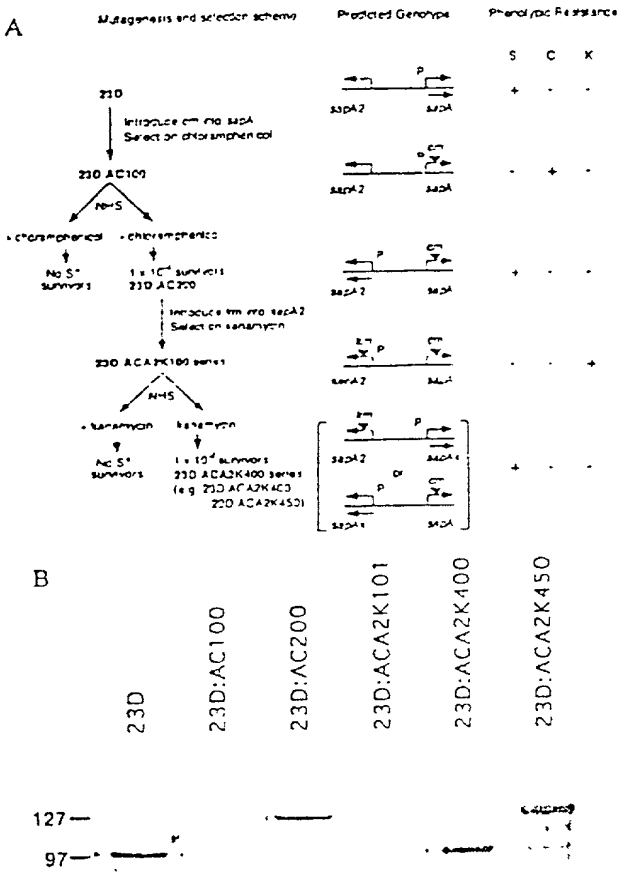
A
Mutagenesis and selection scheme
Predicted Genotype
Phenotypic Resistance
S
C
K
23D
Introduce cm into sapA
Select on chloramphenicol
23D AC100
NaHS
+ chloramphenicol
+ chloramphenicol
No S+ survivors
1 x 10⁷ survivors
23D AC200
Introduce km into sapA2
Select on kanamycin
23D ACA2K100 series
NaHS
+ kanamycin
kanamycin
No S+ survivors
1 x 10⁷ survivors
23D ACA2K400 series
(e.g. 23D ACA2K403
23D ACA2K450)
sapA2
sapA
P
or
B
23D
23D:AC100
23D:AC200
23D:ACA2K101
23D:ACA2K400
23D:ACA2K450
127
97

FIGURE 7

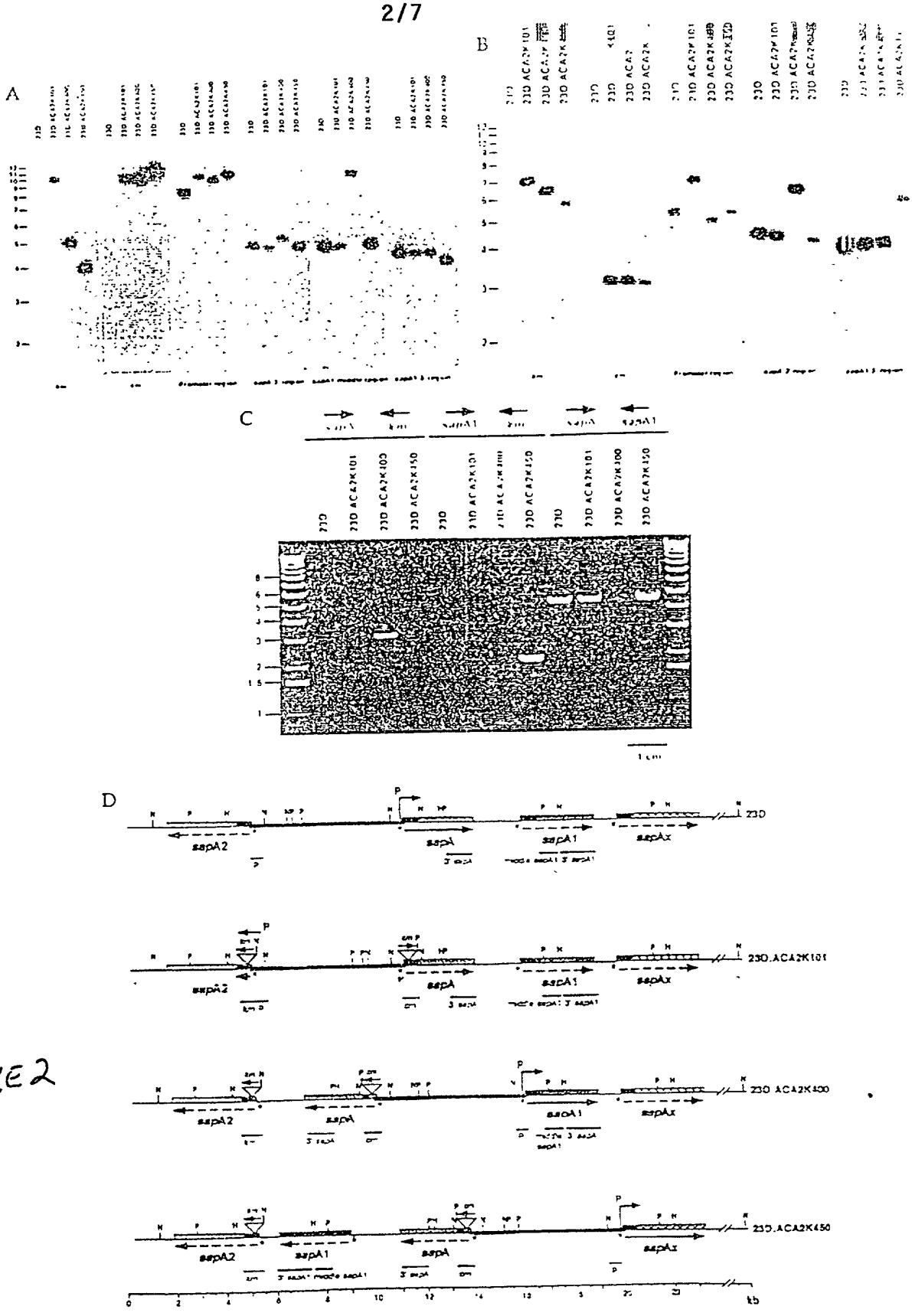

FIGURE 2

FIG 2 (*Legend appears at the bottom of the opposite page.*)

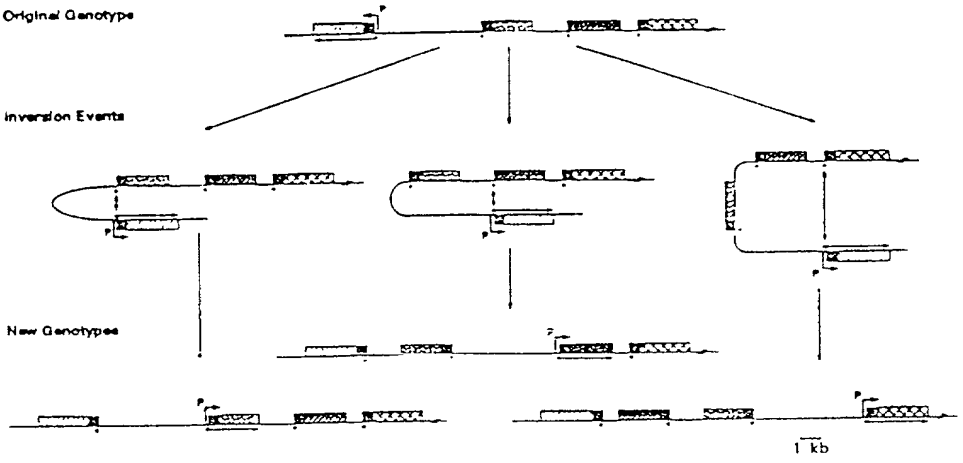

FIGURE 3

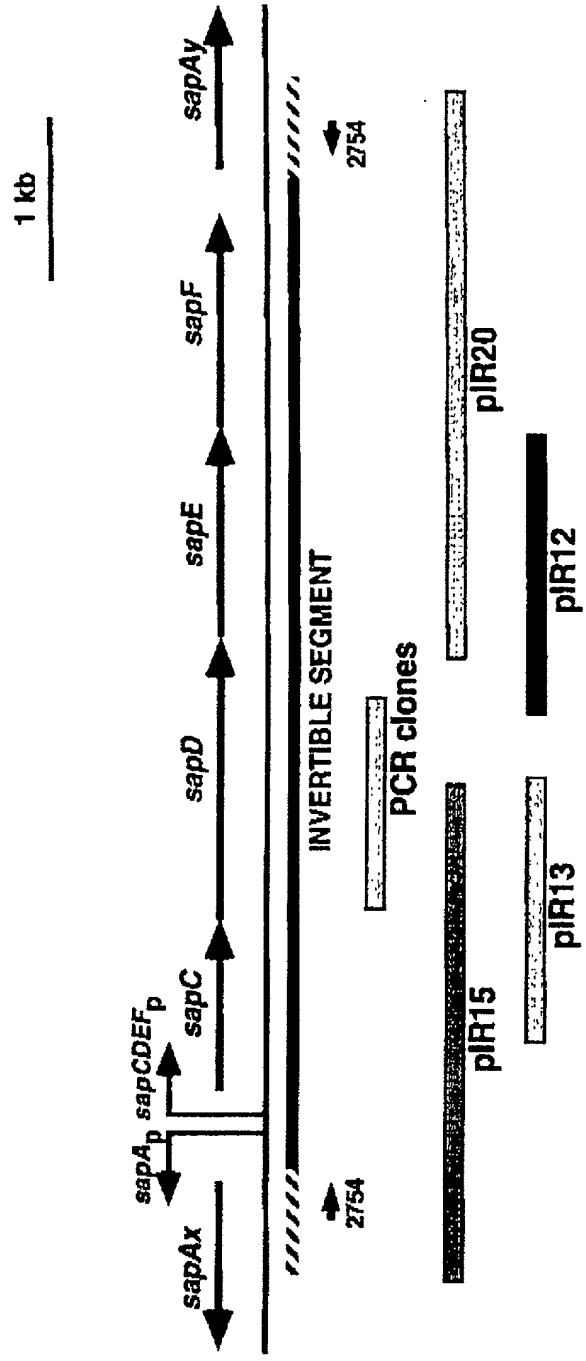
1 kb
sapAy
2754
sapF
sapE
sapD
sapC
sapCDEF$_p$
sapA$_p$
sapAx
2754
INVERTIBLE SEGMENT
PCR clones
pIR20
pIR12
pIR13
pIR15

Figure 4

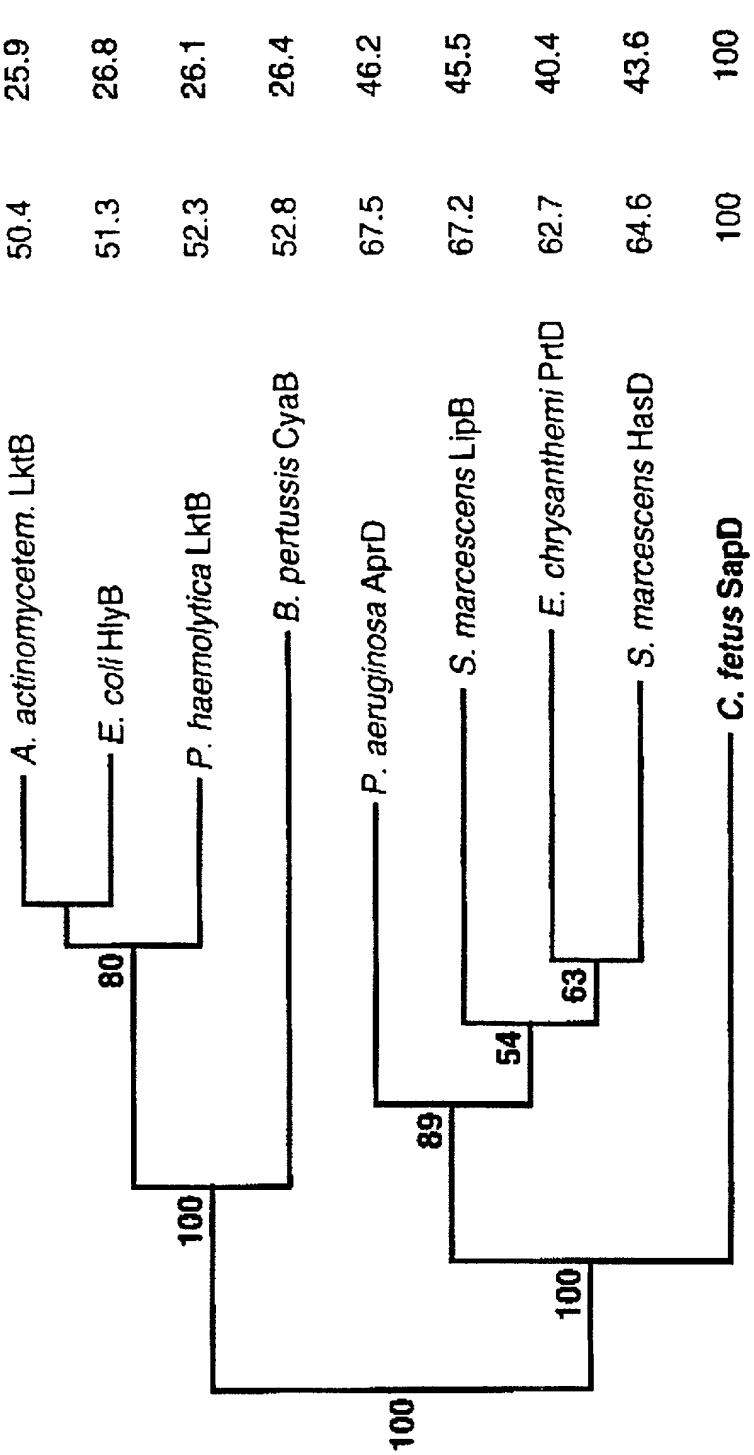
% Sim
% ID
A. actinomycetem. LktB 50.4 25.9
E. coli HlyB 51.3 26.8
P. haemolytica LktB 52.3 26.1
B. pertussis CyaB 52.8 26.4
P. aeruginosa AprD 67.5 46.2
S. marcescens LipB 67.2 45.5
E. chrysanthemi PrtD 62.7 40.4
S. marcescens HasD 64.6 43.6
C. fetus SapD 100 100
80
100
100
89
54
63
100

Figure 5

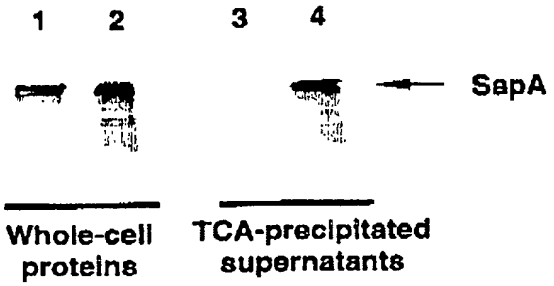
1
2
3
4
SapA
Whole-cell proteins
TCA-precipitated supernatants

Figure 6

Use of *C. fetus* as a live vector for antigen delivery to the mucosal immune system

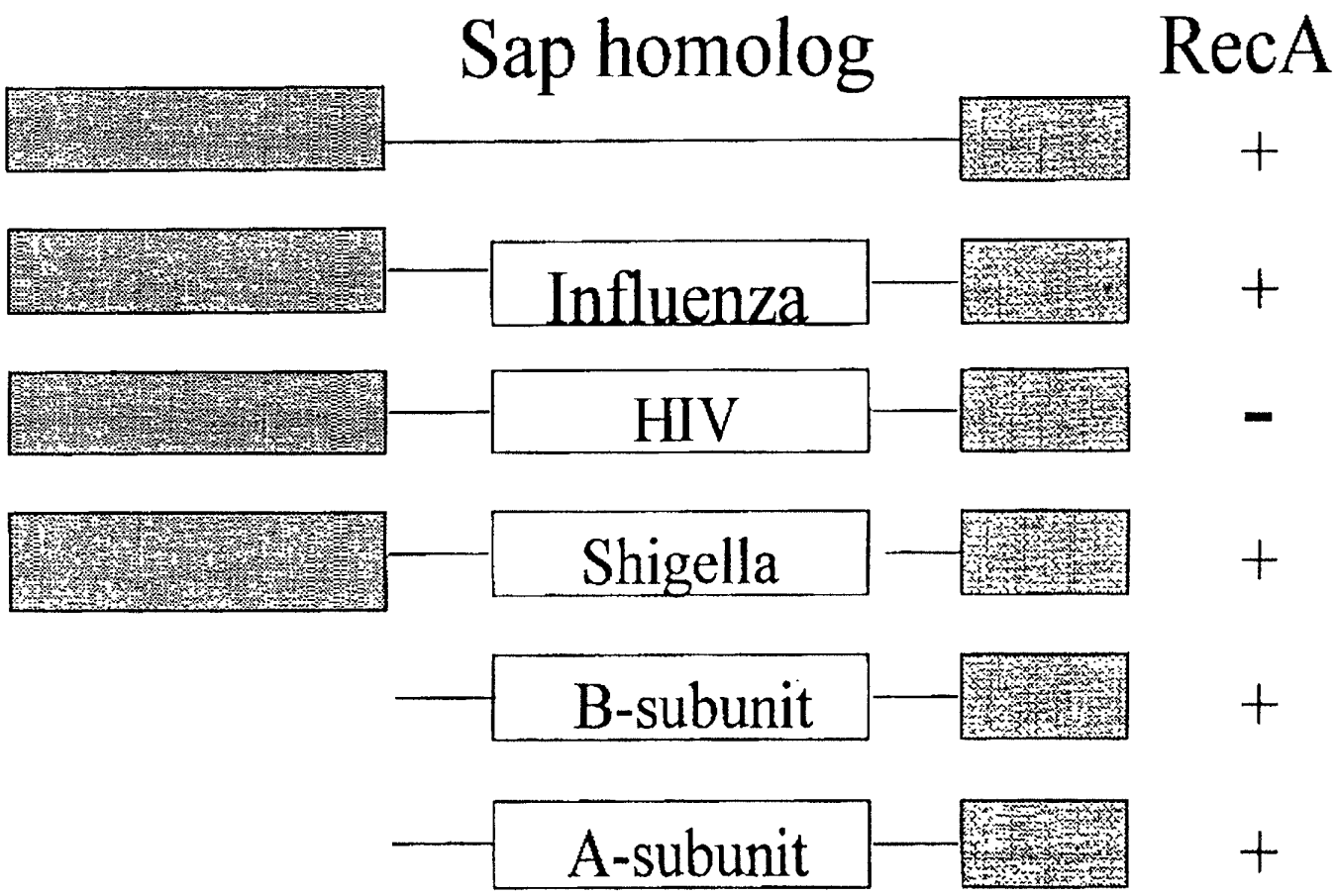

Figure 7

WO 98/33386
PCT/US98/01780
09/355793

DOCKET NO: D5979

COMBINED DECLARATION AND POWER OF ATTORNEY

I, **Stuart A. Thompson**, hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and joint inventor, together with **Martin Blaser** and **Joel Dworkin**, of the subject matter which is claimed and for which a patent is sought under 35 U.S.C. 371 on the invention entitled, **Method of Delivering Antigens for Vaccination with a Live Vector**; the specification of which was filed January 30, 1998 as PCT/US98/01780 and which claims benefit of priority under 35 U.S.C. 119(e) of US provisional application 60/036,321 filed January 31, 1997.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Dr. Benjamin Adler, Registration No. 35,423. Address all telephone calls to Dr. Benjamin Adler at telephone number 713/777-2321. Address correspondence to Dr. Benjamin Adler, McGREGOR & ADLER, LLP, 8011 Candle Lane, Houston, TX 77071.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Full Name of Inventor: **Stuart A. Thompson**

Inventor's Signature: _____ Date: _____

Residence: 2423 Claylick Rd., Whites Creek, TN 37189

Citizen of: United States of America

Post Office Address: 2423 Claylick Rd., Whites Creek, TN 37189

DOCKET NO: D5979

COMBINED DECLARATION AND POWER OF ATTORNEY

I, **Martin Blaser**, hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and joint inventor, together with **Stuart A. Thompson** and **Joel Dworkin**, of the subject matter which is claimed and for which a patent is sought under 35 U.S.C. 371 on the invention entitled, **Method of Delivering Antigens for Vaccination with a Live Vector**; the specification of which was filed January 30, 1998 as PCT/US98/01780 and which claims benefit of priority under 35 U.S.C. 119(e) of US provisional application 60/036,321 filed January 31, 1997.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Dr. Benjamin Adler, Registration No. 35,423. Address all telephone calls to Dr. Benjamin Adler at telephone number 713/777-2321. Address correspondence to Dr. Benjamin Adler, McGREGOR & ADLER, LLP, 8011 Candle Lane, Houston, TX 77071.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Full Name of Inventor: **Martin Blaser**

Inventor's Signature: _____ Date: _____

Residence: 733 Darden Place, Nashville, TN 37205

Citizen of: United States of America

Post Office Address: 733 Darden Place, Nashville, TN 37205

DOCKET NO: D5979

COMBINED DECLARATION AND POWER OF ATTORNEY

I, **Joel Dworkin**, hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and joint inventor, together with **Martin Blaser** and **Stuart A. Thompson**, of the subject matter which is claimed and for which a patent is sought under 35 U.S.C. 371 on the invention entitled, **Method of Delivering Antigens for Vaccination with a Live Vector**; the specification of which was filed January 30, 1998 as PCT/US98/01780 and which claims benefit of priority under 35 U.S.C. 119(e) of US provisional application 60/036,321 filed January 31, 1997.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Dr. Benjamin Adler, Registration No. 35,423. Address all telephone calls to Dr. Benjamin Adler at telephone number 713/777-2321. Address correspondence to Dr. Benjamin Adler, McGREGOR & ADLER, LLP, 8011 Candle Lane, Houston, TX 77071.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Full Name of Inventor: **Joel Dworkin**

Inventor's Signature: _____ Date: _____

Residence: 718 Evans Ave., Kirkwood, MO 63122

Citizen of: United States of America

Post Office Address: 718 Evans Ave., Kirkwood, MO 63122

DOCKET NO: D5979

COMBINED DECLARATION AND POWER OF ATTORNEY

I, **Martin Blaser**, hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and joint inventor, together with **Stuart A. Thompson** and **Joel Dworkin**, of the subject matter which is claimed and for which a patent is sought under 35 U.S.C. 371 on the invention entitled, **Method of Delivering Antigens for Vaccination with a Live Vector**; the specification of which was filed January 30, 1998 as PCT/US98/01780 and which claims benefit of priority under 35 U.S.C. 119(e) of US provisional application 60/036,321 filed January 31, 1997.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Dr. Benjamin Adler, Registration No. 35,423. Address all telephone calls to Dr. Benjamin Adler at telephone number 713/777-2321. Address correspondence to Dr. Benjamin Adler, McGREGOR & ADLER, LLP, 8011 Candle Lane, Houston, TX 77071.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

1-00

Full Name of Inventor: **Martin Blaser**

Inventor's Signature: _____ Date: 9/8/99

Residence: 733 Darden Place, Nashville, TN 37205 TN

Citizen of: United States of America

Post Office Address: 733 Darden Place, Nashville, TN 37205

DOCKET NO: D5979

COMBINED DECLARATION AND POWER OF ATTORNEY

I, **Joel Dworkin**, hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and joint inventor, together with **Martin Blaser** and **Stuart A. Thompson**, of the subject matter which is claimed and for which a patent is sought under 35 U.S.C. 371 on the invention entitled, **Means for Delivering Antigens for Vaccination with a Live Vector**; the specification of which was filed January 30, 1998 as PCT/US98/01780 and which claims benefit of priority under 35 U.S.C. 119(e) of US provisional application 60/036,321 filed January 31, 1997.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Dr. Benjamin Adler, Registration No. 35,423. Address all telephone calls to Dr. Benjamin Adler at telephone number 713/777-2321. Address correspondence to Dr. Benjamin Adler, McGREGOR & ADLER, LLP, 8011 Candle Lane, Houston, TX 77071.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful *false statements* and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

200 Full Name of Inventor: Joel Dworkin, MD, Ph.D

Inventor's Signature: [signature] Date: 8/6/99

Residence: 718 Evans Ave., Kirkwood, MO 63122 MO

Citizen of: United States of America

Post Office Address: 718 Evans Ave., Kirkwood, MO 63122

DOCKET NO: D5979

COMBINED DECLARATION AND POWER OF ATTORNEY

I, **Stuart A. Thompson**, hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and joint inventor, together with **Martin Blaser** and **Joel Dworkin**, of the subject matter which is claimed and for which a patent is sought under 35 U.S.C. 371 on the invention entitled, **Method of Delivering Antigens for Vaccination with a Live Vector**; the specification of which was filed January 30, 1998 as PCT/US98/01780 and which claims benefit of priority under 35 U.S.C. 119(e) of US provisional application 60/036,321 filed January 31, 1997.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Dr. Benjamin Adler, Registration No. 35,423. Address all telephone calls to Dr. Benjamin Adler at telephone number 713/777-2321. Address correspondence to Dr. Benjamin Adler, McGREGOR & ADLER, LLP, 8011 Candle Lane, Houston, TX 77071.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

300

Full Name of Inventor: **Stuart A. Thompson**

Inventor's Signature: Stuart A. Thompson Date: 9/9/99

Residence: 890-A North Belair Rd., Evans, Georgia 30809 GA

Citizen of: United States of America

Post Office Address: 890-A North Belair Rd., Evans, Georgia 30809